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<p>(54) Title: INHIBITION OF INFECTION OF MAMMALIAN CELLS BY RESPIRATORY SYNCYTIAL VIRUS  (57) Abstract  The infection of mammalian cells by RSV may be inhibited by native human <math>\beta</math>-casein, a recombinant form of human <math>\beta</math>-casein, and hydrolysates of both. The human <math>\beta</math>-casein or hydrolysate may be contained in a liquid enteral nutritional product such as an infant formula. The enteral nutritional product may be used, for example, in the prevention and treatment of respiratory tract infection in infants. The human <math>\beta</math>-casein or hydrolysate may also be administered as a throat spray or nasally using drops or a spray.</p>		

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## INHIBITION OF INFECTION OF MAMMALIAN CELLS BY RESPIRATORY SYNCYTIAL VIRUS

The present invention relates generally to inhibiting the infection of mammalian cells by Respiratory Syncytial Virus, and more specifically to the use of native or recombinant human  $\beta$ -casein and hydrolysates thereof for inhibiting the infection of mammalian cells by Respiratory Syncytial Virus.

Respiratory Syncytial Virus (RSV) is the single most frequent cause of acute respiratory tract infection in infants and children. Infants less than six months of age are most frequently and seriously affected. In most immunologically normal subjects, infection with RSV is limited to the respiratory mucosa, and is associated with the development of bronchiolitis, pneumonia and reactive airway disease. RSV infection in immunocompromised subjects has until recently been associated with increased mortality in infants and increased morbidity in other age groups. It has recently been reported in PEDIATRIC NOTES, Vol. 18, No. 14, January 27, 1994, that periods of high incidence of acute respiratory disease and numbers of deaths in elderly people were followed within 2-3 weeks by reports of high numbers of RSV or influenza virus isolates. The analyses indicate that RSV is as important as influenza viruses in causing morbidity and deaths among the elderly.

It has been reported that some respiratory disease may be prevented by breast feeding, and that "bronchiolitis of infants due to respiratory syncytial virus is less frequent in breast fed than in artificially fed infants". While human breast milk can contain antibodies to RSV, it has been found that milk also has antiviral activity that is not due to antibodies. It has been theorized that this effect "may be produced by certain polysaccharides which are found on a number of different molecular constituents of milk." Tyrrell, "BREAST FEEDING AND VIRUS INFECTIONS", THE IMMUNOLOGY OF INFANT FEEDING, edited by A. W. Wilkinson, Plenum Press, New York, NY pages 55-62 (1981).

Okamoto, et al., "Antiviral Factors in Human Milk: Implications in Respiratory Syncytial Virus Infection", ACTA PAEDIATRICA SCANDANAVICA SUPPLEMENT, 351:137-143 (1989) disclose that while the mechanisms of protective immunity to RSV had not been clearly defined, immunity acquired

transplacentally or via breast feeding has been suggested to reduce the risk of lower respiratory tract disease. However, this publication focuses upon the role of antibodies transmitted in breast milk or the possible role of breast milk in modulating an infant's RSV immune response.

Laegreid et al., "Neutralizing Activity in Human Milk Fractions against Respiratory Syncytial Virus", ACTA PAEDIATRICA SCANDANAVICA, 75:696-701 (1986) reports a study which confirms that human milk may contain RSV-neutralizing activity of a non-immunoglobulin nature as well as RSV-specific antibody. However, the identity and mechanism of the non-immunoglobulin anti-RSV component of human milk is not identified. It is important though to note that Laegreid et al. disclose that RSV-neutralizing components from breast milk may reach an infant's respiratory tract directly as a result of regurgitation and inhalation of milk during and after feeding. The mucosa of the respiratory tract may gain direct protection in this way.

WO 91/06308 filed by Andersson et al. for "ANTIBACTERIAL COMPOSITION", and a published article by the same authors (Aniansson et al., "Anti-adhesive activity of human casein against Streptococcus pneumonia and Haemophilus influenzae", MICROBIAL PATHOGENESIS, 8:315-323 (1990) disclose the use of a milk fraction having a molecular weight of at least 5,000 daltons for "therapeutic prophylactic, and/or diagnostic use in infections caused by *S. pneumoniae* and/or *H. influenzae*", but it is suggested in these publications that the beneficial effect is provided by kappa-casein. However, the present invention relates to the use of native or recombinant human  $\beta$ -casein and hydrolysates of both to inhibit RSV infections.

WO93/04172 relates to a DNA sequence encoding human  $\beta$ -casein, but does not disclose the capacity of either native or recombinant human  $\beta$ -casein to inhibit the attachment of RSV to human cells.

WO91/08675 discloses an infant formula which contains recombinant forms of both human alpha-lactalbumin and human  $\beta$ -casein. However, this publication discloses only that these human milk proteins will "give a simulated human mother's milk formula that does not exhibit the allergenic properties associated with formulas based on cow or other foreign protein." (page 3, lines 20-22). The use of human  $\beta$ -casein to inhibit the attachment of RSV to human cells is not taught or suggested in said publication.

The two assays (a HEp-2 cell assay and a LLC-MK2 cell assay) which were used for determining the bioactivity of  $\beta$ -casein are described below. These assays have not been published heretofore, although the HEp-2 cell

## 3.

assay was based upon established methodology.

### MATERIALS USED IN BOTH ASSAYS

#### Native Human $\beta$ -Casein

$\beta$ -casein isolated from human milk was purchased from Symbicom AB, P.O. Box 1451, S-901 24 Umea, Sweden.

#### Recombinant Human $\beta$ -Casein

Applicants obtained  $\beta$ -casein cDNA and the expression system from Symbicom AB, P.O. Box 1451, S-901 24 Umea, Sweden. The human  $\beta$ -casein cDNA used had been previously cloned and sequenced by Lonnerdal et al., Cloning and sequencing of a cDNA encoding human milk  $\beta$ -casein. (SEQ.ID NO: 1:) *Federation of European Biochemical Societies Letters* 269, 153-156 (1990). The recombinant human  $\beta$ -casein was obtained from *E. coli* and purified according to the method of Hansson et al., "Expression of Human Milk  $\beta$ -Casein in *Escherichia coli*: Comparison of Recombinant Protein with Native Isoforms". *Protein Expression and Purification* 4, 373-381 (1993). To express human  $\beta$ -casein in *E.coli*,  $\beta$ -casein cDNA was cloned under control of a T7 promoter in two different expression vectors. One vector, pS26, was designed for intracellular expression. The other vector, pS28, has a signal sequence for extracellular expression. The procedure followed was substantially that described by Hansson et al.

Human  $\beta$ -casein cDNA was isolated by Hansson et al. as a 1.1-kb *EcoRI* fragment from a human lambda gt mammary gland library, and was subcloned into pUC19, which was designated pS21. The cDNA was modified by introduction of synthetic oligonucleotides in the 5' and 3' termini. To introduce a suitable cloning site in the 5' end, *NdeI*, a translational start, was inserted in front of the sequence encoding mature human  $\beta$ -casein. To adapt the initial part of the translated sequence to *E.coli* codon usage, six synthetic oligonucleotides were constructed and ligated. Also, *PstI* and *EcoRI* sites were inserted in front of the *NdeI* site. The sequence of the synthetic fragment was 5'-CTGCAGAATTCATATGCGT GAAACCATCGAATCCCTGAGCTCGAGCGAAGAATCGATCACCGAATACAAAAAGTTGAAAAAGTTAAACACG AGGACCAGGATCC-3'. (SEQ ID NO: 2:) The protein encoding sequence is underlined. The synthetic fragment was cloned into *PstI/BamHI*-digested pUC19 resulting in plasmid pS24. To insert the rest of the  $\beta$ -casein

encoding sequence, a 303-bp *AccI/BglII* fragment was isolated and cloned into a pUC18 derivative and designated plasmid pS22. Four synthetic oligonucleotides containing the sequence encoding the carboxy-terminal end and translation stop followed by *BamHI* and *EcoRI* sites were constructed resulting in the sequence 5' AGATCTACCCGTGA  
CTCAGCCACTTGCCCCAGTTCATAACCCCATAGTGTCTAATAAGGATCCGAATTC-3'.

(SEQ ID NO: 3:) where the protein encoding sequence is underlined. The synthetic fragment was cloned into *BglII/EcoRI* digested pS22, resulting in plasmid pS23. To obtain the recombinant modified  $\beta$ -casein encoding fragment, three fragments were ligated: an 89-bp *PstI/AvaII* fragment from pS24; a 197-bp *AvaII/AccI* fragment from pS21; and *PstI/AccI* digested pS23. The resulting plasmid pS25 was digested with *NdeI/BamHI* and a 641-bp fragment was isolated and cloned into the vector pET-3a. The resulting expression vector was designated pS26.

In order to construct a vector mediating extracellular expression, the *E. coli* signal sequence of the enterotoxin STII gene was introduced in front of the  $\beta$ -casein encoding sequence. A modified STII sequence with *NcoI*- and *NdeI*-compatible ends and an internal *ClaI* site was obtained by using a synthetic oligonucleotide, 5'-CATGAAAAAGAATATCGCATTTCTTCTTGCATCGATGTTTCGTTT  
TTTCTATTGCTACAAATGCATATG-3' (SEQ ID NO: 4:). To insert the signal sequence in front of the  $\beta$ -casein encoding sequence, pS25 was digested with *AvaI/EcoRI* and a 619-bp fragment was isolated. This fragment was ligated with a synthetic oligonucleotide fragment, 5'-CATATGCACGTGAAACCATCGAATCCCTGAGCTCGAG-3' (SEQ ID NO: 5:), and *NdeI/EcoRI*-digested pUC19. The resulting plasmid was designated pS27. The final expression vector, pS28, was constructed by ligating three fragments: a 700-bp *NdeI/HindIII*  $\beta$ -casein fragment isolated from pS27, the STII signal sequence, and a *NcoI/HindIII*-digested pACAT7 vector.

The expression vectors pS26 and pS28 were used to transform *E. coli* strains BL21(DE3), BL21(DE3)pLysS, and BL21(DE3)pLysE. The bacteria were grown in Luria Broth medium containing 50  $\mu$ g/ml carbenicillin, and when BL21(DE3)pLysS and BL21(DE3)pLysE were used the medium was supplemented with 25  $\mu$ g/ml chloramphenicol.

For induction of expression the cultures were grown to a density yielding an optical density (OD) of 0.6 at a wavelength of 600 nanometers (OD<sub>600</sub>), then 0.4 mM IPTG was added to induce the T7 system. The cells were

harvested about 90 minutes after induction.

Recombinant  $\beta$ -casein was isolated using standard procedures. The inducible T7-based expression system resulted in high-level expression of recombinant  $\beta$ -casein. Bacteria were harvested and the cells pelleted by centrifugation. The supernatant contained the periplasmic proteins and the pellet the cytoplasmic fraction. The recombinant proteins obtained were compared with native  $\beta$ -casein, which had been purified by standard methods including either ion-exchange chromatography followed by reversed-phase HPLC or gel filtration. Recombinant and native  $\beta$ -casein were compared by standard biochemical techniques comprising SDS-PAGE, Western blotting, amino acid analysis, peptide mapping, phosphate analysis, and mass spectrometry. Recombinant  $\beta$ -casein expressed in *E.coli* was found to comigrate with full-length, nonphosphorylated native human  $\beta$ -casein, which is one of seven native isoforms.

Recombinant human  $\beta$ -casein has also been expressed in *S.cerevisiae* using the pYES 2.0 vector (Invitrogen Corp., San Diego, CA), but the expression level was approximately 10% of that obtained in *E.coli*. However, Hansson et al. found that *S. cerevisiae* appeared to express phosphorylated human milk  $\beta$ -casein.

$\beta$ -Casein Hydrolysates

The human  $\beta$ -casein (both native and recombinant) was digested using the specific endoproteinase GLU-C (Sigma, sequencing grade) which catalyzes the hydrolysis of peptide bonds at the C-terminal of glutamic acid residue. After monitoring the digest using high pressure liquid chromatography, an enzyme to protein ratio of 1:100 (weight/weight) was chosen for a 30 hour digestion at 37°C in 0.1 M  $\text{NH}_4\text{HCO}_3$ , pH 7.8. These digests were dried and resuspended in appropriate buffers prior to use in the assays discussed above.



INHIBITION OF HUMAN RSV INFECTION OF HEp-2 CELLS

The Long strain of RSV was grown in HEp-2 cells under Eagle minimal essential medium (MEM) with 2% fetal bovine serum (FBS). The virus was harvested at a cytopathic effect (CPE) of 3 to 4+, sonicated for 10 seconds at 50% power with a Microson ultrasonic bell disrupter (Heat Systems - Ultrasonics, Inc., Farmingdale, N.Y.), divided into portions and stored at -70°C. The same preparation of virus was used for a series of tests, but a fresh sample was used for each test run.

The neutralization tests were performed in 96-well, flat-bottomed, tissue culture, microtiter plates (catalog no. 3596; Costar, Cambridge, Mass.). Serum or a monoclonal antibody (MAb) in the form of ascites fluid, which had been heat inactivated at 56°C for 30 min., was added to duplicate wells and serial fourfold dilutions were performed in the microtiter plates. All dilutions were in MEM-2% FBS, and the final volume was 75  $\mu$ l per well. Approximately 60 50% tissue culture infective doses of virus in 25  $\mu$ l of MEM-2% FBS then were added to each well, and the mixture was incubated for 2 h at 4°C.

Approximately 15,000 HEp-2 cells in 100  $\mu$ l of MEM-5% FBS were added to each well, and the plates were wrapped in cellophane and incubated at 35.5°C in a humidified CO<sub>2</sub> incubator for 3 days. The plates were fixed by aspirating the contents of the wells, washing three times with phosphate-buffered saline (PBS) at pH 7.2 with 0.5% Tween 20, adding 75  $\mu$ l of an 80% (vol/vol) solution of acetone-PBS, and incubating for 15 min at 4°C. After the incubation period, the contents were aspirated, and the plates were air dried.

The Enzyme Linked Immuno Sorbent Assay (ELISA) was performed on the same day as the fixation, or the plates were stored overnight at 4°C and the ELISA was performed on the next day. For the ELISA, the wells were precoated with 200  $\mu$ l of PBS with 0.5% gelatin for 30 min at 35°C, the contents were aspirated, the wells were washed with PBS (pH 7.2)-0.5% Tween 20 and 75  $\mu$ l of bovine anti-RSV serum (BaRSV) Burroughs Wellcome Co., Research Triangle Park, N.C.) diluted in PBS 0.5% gelatin plus 0.5% Tween 20 and 2% normal goat serum was added and incubated for 1 hour at 35.5°C. The contents were aspirated, the wells were washed, and 75  $\mu$ l of peroxidase-conjugated, goat anti-bovine immunoglobulin G (IgG) (Kirkegaard and Perry

Laboratories, Inc., Gaithersburg, MD) diluted in PBS-0.5% gelatin-0.5% Tween 20-2% normal goat serum was added and incubated for 1 hour at 35.0°C. The contents of the wells were aspirated again, the wells were washed, and 125  $\mu$ l of substrate (0.4 mg of *o*-phenylenediamine dihydrochloride per ml 0.015% H<sub>2</sub>O<sub>2</sub>) in 0.15 M citrate phosphate buffer (pH 5.5) was added and incubated at room temperature for 40 to 45 min. The reaction was stopped with 3.5 M HCl, and the A<sub>490</sub> was read with a microplate reader. Each dilution of antibody was run in duplicate, and each plate included control wells with uninfected cells, a back titration, i.e. titration of the virus inoculum in MEM-2% FBS, and a titration of a preimmune or nonneutralizing antibody. An absorbance reading of greater than or equal to 3 standard deviations above the mean of 15 control wells was considered to be evidence of virus replication. The dilutions of BaRSV (1:1,000) and goat anti-bovine IgG (1:5,000) used through the study initially was determined by checkerboard titrations.

This assay was based upon the disclosure of Anderson et al., "Microneutralization Test for Respiratory Syncytial Virus Based on an Enzyme Immunoassay", JOURNAL OF CLINICAL MICROBIOLOGY, December 1985, pages 1050-1052.

RESULTS FROM HEP-2 CELL ASSAY

The human and bovine  $\beta$ -casein solutions were prepared first in 20 mM ethanolamine, 6 M urea, pH 9.5 and then washed twice in PBS by ultrafiltration using Centricon membrane filters (Amicon, MA) with a cut-off of 3,000 daltons. After resuspending in appropriate buffer for the HEP-2 cell assay described above, these samples were tested in the assay. Experiments with different designated numbers were performed in different days. As shown in Table 1, human  $\beta$ -casein caused an inhibition of infection/virus replication of 50% or more at concentrations of 0.4 mg/ml or greater. It should be noted that when referring to Table 1, a higher percent inhibition indicates a higher level of bioactivity of the "AGENT", and a lower percent inhibition indicates a lower level of activity of the "AGENT". Bovine  $\beta$ -casein was not significantly active even at 1.6 mg/ml. These results indicated that  $\beta$ -casein from human milk has different bioactivity compared to the bovine milk  $\beta$ -casein.

TABLE 1  
INHIBITION OF HUMAN RSV INFECTION OF HEp-2 CELLS

<u>AGENT USED</u>	<u>CONC.</u> (mg/ml)	<u>PERCENT INHIBITION</u>
$\beta$ -Casein Isolated from Human Milk	1.6	>90
	0.8	>90
	0.4	>90
Bovine $\beta$ -casein	1.6	0
	0.8	0
	0.4	0

Data shown are average of three replicates.

INHIBITION OF HUMAN RSV INFECTION OF LLC-MK2 CELLS

The RSV inhibition assay quantitatively determines the ability of a test reagent (antibody or other bioactive compound) to inhibit the infection of monkey kidney cells (LLC-MK2) (ATCC CCL 7) in microtiter plates. Infected cells were identified using an immunoperoxidase method. The method is described briefly below.

LLC-MK2 cells were seeded into fibronectin treated Costar microtiter plates ( $5.0 \times 10^3$  cells per well) and incubated for 3-4 days prior to use in the infectivity reduction assay. On the day of assay, the Long strain of RSV was diluted in MEM to 10-20,000 infected cell units (ICU/mL), and added to an equal volume (200  $\mu$ L) of serially diluted sample preparations at suitable concentration (ex. 0.5, 1.0, and 2.0 mg casein/mL). Mixtures of diluted test samples and virus were then incubated for 2 hours at 4°C prior to adding to LLC-MK2 cells. Prior to addition of the diluted sample-virus mixtures to microtiter plates, culture medium was removed and the monolayers rinsed one time with MEM. All diluted sample-virus mixtures were tested in triplicate wells. The diluted sample-virus mixtures were allowed to absorb to LLC-MK2 monolayers for 2 hours at 37°C in a humidified CO<sub>2</sub> incubator. Following incubation, 150  $\mu$ l of MEM was added to all wells and the plates incubated at 37°C for 16 hours in the CO<sub>2</sub> incubator. After overnight incubation, the culture medium was removed and the monolayers fixed with cold ethanol. After fixing, microtiter plates were rinsed once with 200  $\mu$ l Dulbecco's PBS per well, and bovine anti-RSV antibody (200  $\mu$ l) was added to all wells. Following a 30 minute incubation at room temperature and three rinses with PBS/0.5% chick albumen (PBS/CEA), peroxidase labeled rabbit anti-bovine IgG was added to all wells and incubated at room temperature for 30 minutes. Microtiter plates were then rinsed 3 times with PBS/CEA and diaminobenzadine substrate added and incubated for 20 minutes. Plates were then rinsed as above with PBS/CEA, and the number of stained RSV-infected cells per well determined using an inverted microscope.

RESULTS FROM LLC-MK2 CELL ASSAY

The proteins described in Table 1 were also tested in this assay for inhibition of RSV infection. Results from this assay are shown in Table 2. Once again, native human milk  $\beta$ -casein was found to be active at concentrations of 1 mg/ml or greater while bovine  $\beta$ -casein was not significantly active. The GLU-C hydrolysates of both native and recombinant human  $\beta$ -casein were active at concentrations of 0.75 mg/ml and higher. Hence these results indicated that the recombinant human  $\beta$ -casein, native human  $\beta$ -casein and their hydrolysates inhibit RSV infection of both HEP-2 mammalian cells and LLC-MK2 mammalian cells.

TABLE 2

INHIBITION OF HUMAN RSV INFECTION OF LLC-MK2 CELLS

<u>AGENT USED</u>	<u>CONC.</u> (mg/ml)	<u>PERCENT INHIBITION</u>
$\beta$ -Casein Isolated from Human Milk	1.5	87
	1	69
	0.75	33
	0.38	20
Bovine $\beta$ -casein	1	21
	0.5	23
	0.25	6
Hydrolysate of $\beta$ -casein Isolated from Human Milk	1.5	99
	0.75	77
	0.38	60
Hydrolysate of Recombinant Human $\beta$ -Casein	1.5	84
	0.75	42
	0.38	25
GLU-C Enzyme Control	.025	64
	.0125	27
	.0068	19

Data shown are average of four replicates.

It has been concluded from the foregoing experiments that  $\beta$ -casein isolated from human milk, a recombinant form of the  $\beta$ -casein contained in human milk, and hydrolysates of both, inhibits the infection of mammalian cells by *RSV*. Furthermore, inasmuch as *RSV* has been identified in the literature as being associated with respiratory tract infection, it has been concluded that the above identified forms of human  $\beta$ -casein may be employed in the prevention and treatment of respiratory tract infection in humans, especially in human infants. In view of the therapeutic effect of enterally ingested human milk containing human  $\beta$ -casein upon respiratory tract infection, it is concluded that the above identified forms of human  $\beta$ -casein have a therapeutic benefit when enterally (orally) ingested.

The therapeutic effects described in the preceding paragraph may be provided by an enteral liquid nutritional product, such as infant formula, comprising one or more proteins not contained in human milk in combination with a therapeutically effective amount of at least one of the forms of human  $\beta$ -casein described in the preceding paragraph. It is further concluded that the infection of mammalian cells by *RSV* may be inhibited by administering via a nasal passageway, or as a throat spray, a formulation containing a therapeutically effective amount of at least one of the forms of human  $\beta$ -casein identified in the preceding paragraph. Such a nasally administered formulation may be in the form of either drops or a spray.

The enteral nutritional, throat spray and nasal products and methods are believed to be effective in inhibiting the infection of mammalian cells by *RSV* because the interaction of the human  $\beta$ -casein and *RSV* is believed to occur via direct contact rather than following digestion and absorption of the  $\beta$ -casein.

It is believed that the above identified forms of human  $\beta$ -casein may be incorporated into any standard or specialized enteral liquid nutritional product containing at least one protein not found in human milk, such as bovine milk based or soy based infant formulas, and other beverages consumed by young children. In a preferred embodiment no proteins or hydrolysates thereof found in human milk, other than  $\beta$ -casein, are contained in the liquid enteral nutritional product. Such a product has utility in the treatment and prevention of respiratory tract infection in human infants.

While preferred embodiments of the invention have been disclosed, it will be apparent to those skilled in the art that various changes and



modifications may be made therein without deviating from the spirit or scope of this invention.

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- (iii) NUMBER OF SEQUENCES:** 5
- (iv) CORRESPONDENCE ADDRESS:**
  - (A) ADDRESSEE:** Lonnie R. Drayer  
ROSS Products Division  
Abbott Laboratories
  - (B) STREET:** 625 Cleveland Avenue
  - (C) CITY:** Columbus
  - (D) STATE:** Ohio
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  - (F) ZIP:** 43215
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  - (B) TELEFAX: (614) 624-3074
  - (C) TELEX: None
- (2) INFORMATION FOR SEQ ID NO: 1
  - (i) SEQUENCE CHARACTERISTICS:
    - (A) LENGTH: 1065 base pairs
    - (B) TYPE: Nucleic acid
    - (C) STRANDEDNESS: Single
    - (D) TOPOLOGY: Unknown
  - (ii) MOLECULE TYPE: Cloned cDNA representing the product of a human genomic DNA segment
    - (A) DESCRIPTION: Human milk  $\beta$ eta-casein
  - (iii) HYPOTHETICAL:
  - (iv) ANTI-SENSE:
  - (v) FRAGMENT TYPE:
  - (vi) ORIGINAL SOURCE: Human
    - (A) ORGANISM: Homo sapiens
    - (B) STRAIN:
    - (C) INDIVIDUAL ISOLATE:

- (D) DEVELOPMENTAL STAGE: Adult
- (E) HAPLOTYPE:
- (F) TISSUE TYPE: Mammary gland
- (G) CELL TYPE:
- (H) CELL LINE:
- (I) ORGANELLE:
- (vii) IMMEDIATE SOURCE: Human Mammary Gland
  - (A) LIBRARY:
  - (B) CLONE:
- (viii) POSITION IN GENOME:
  - (A) CHROMOSOME/SEGMENT:
  - (B) MAP POSITION:
  - (C) UNITS:
- (ix) FEATURE:
  - (A) NAME/KEY:
  - (B) LOCATION:
  - (C) IDENTIFICATION METHOD: DNA sequencing and restriction analysis
  - (D) OTHER INFORMATION: The encoded product of nucleotide SEQ ID NO: 1 is the human milk protein,  $\beta$ -casein.
- (x) PUBUBLICATION INFORMATION:
  - (A) AUTHORS: B. Lonnerdal, et al.
  - (B) TITLE: Cloning and sequencing of a cDNA encoding human milk beta-casein.
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- (K) RELEVANT RESIDUES:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1

CGG ATG AAG GTC CTC ATC CTC GCC TGC CTG GTG GCT CTT GCT CTT	45
GCA AGG GAG ACC ATA GAA AGC CTT TCA AGC AGT GAG GAA TCT ATT	90
ACA GAA TAC AAG AAA GTT GAG AAG GTT AAA CAT GAG GAC CAG CAG	135
CAA GGA GAG GAT GAA CAC CAG GAT AAA ATC TAC CCC TCT TTC CAG	180
CCA CAG CCT CTG ATC TAT CCA TTC GTT GAA CCT ATC CCC TAT GGT	225
TTT CTT CCA CAA AAC ATT CTG CCT CTT GCT CAG CCT GCT GTG GTG	270
CTG CCT GTC CCT CAG CCT GAA ATA ATG GAA GTC CCT AAA GCT AAA	315
GAC ACT GTC TAC ACT AAG GGC AGA GTG ATG CCT GTC CTT AAA TCT	360
CCA ACG ATA CCC TTT TTT GAC CCT CAA ATC CCA AAA CTC ACT GAT	405
CTT GAA AAT CTG CAT CTT CCT CTG CCT CTG CTC CAG CCC TTG ATG	450
CAG CAG GTC CCT CAG CCT ATT CCT CAG ACT CTT GCA CTT CCC CCT	495
CAG CCC CTG TGG TCT GTT CCT CAG CCC AAA GTC CTG CCT ATC CCC	540
CAG CAA GTG GTG CCC TAC CCT CAG AGA GCT GTG CCT GTT CAA GCC	585
CTT CTG CTC AAC CAA GAA CTT CTA CTT AAC CCC ACC CAC CAG ATC	630
TAC CCT GTG ACT CAG CCA CTT GCC CCA GTT CAT AAC CCC ATT AGT	675
GTC TAA GAA GAT TTC AAA GTT AAT TTT CCC TCC TTA TTT TTG AAT	720
TGA CTG AGA CTG GAA ATA TGA TGC CTT TTC CGT CTT TGT ATC ACG	765
TTA CCC CAA ATT AAG TAT GTT TGA ATG AGT TTA TAT GGA AAA AAT	810
GAA CTT TGT CCC TTT ATT TAT TTT ATA TAT TAT GTC ATT CAT TTA	855
ATT TGA AAT TTG ACT CAT GAA CTA TTT ACA TTT TCC AAA TCT TAA	900
TTC AAC TAG TAC CAC AGA AGT TCA ATA CTC ATT TGG AAA TGC TAC	945
AAA CAT ATC AAA CAT ATG TAT ACA AAT TGT TTC TGG AAT TGT GCT	990
TAT TTT TAT TTC TTT AAG AAT CTA TTT CCT TTC CAG TCA TTT CAA	1035
TAA ATT ATT CTT AAG CAT AAA AAA AAA AAA	1065

(2) INFORMATION FOR SEQ ID NO: 2

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 105 base pairs

- (B) TYPE: Nucleic acid
- (C) STRANDEDNESS: Single
- (D) TOPOLOGY: Unknown
- (ii) MOLECULE TYPE: Synthetic oligonucleotide
  - (A) DESCRIPTION:
- (iii) HYPOTHETICAL:
- (iv) ANTI-SENSE:
- (v) FRAGMENT TYPE:
- (vi) ORIGINAL SOURCE: Synthetic Oligonucleotide Sequence
  - (A) ORGANISM:
  - (B) STRAIN:
  - (C) INDIVIDUAL ISOLATE:
  - (D) DEVELOPMENTAL STAGE:
  - (E) HAPLOTYPE:
  - (F) TISSUE TYPE:
  - (G) CELL TYPE:
  - (H) CELL LINE:
  - (I) ORGANELLE:
- (vii) IMMEDIATE SOURCE:
  - (A) LIBRARY:
  - (B) CLONE:
- (viii) POSITION IN GENOME:
  - (A) CHROMOSOME/SEGMENT:
  - (B) MAP POSITION:

## (C) UNITS:

## (ix) FEATURE:

## (A) NAME/KEY:

## (B) LOCATION:

## (C) IDENTIFICATION METHOD: DNA sequencing and restriction analysis

## (D) OTHER INFORMATION: The synthetic oligonucleotide assures adaptation of translation sequence to E. coli codon usage.

## (x) PUBUBLICATION INFORMATION:

## (A) AUTHORS: L. Hansson, et al.

(B) TITLE: Expression of Human Milk  $\beta$ -casein in Escherichia coli: Comparison of Recombinant Protein with Native Isoforms.

## (C) JOURNAL: Protein Expression and Purification

## (D) VOLUME: 4

## (E) ISSUE:

## (F) PAGES: 373 - 381

## (G) DATE: 1993

## (H) DOCUMENT NUMBER:

## (I) FILING DATE:

## (J) PUBLICATION DATE:

## (K) RELEVANT RESIDUES:

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2

CTG CAG AAT TCA TAT GCG TGA AAC CAT CGA ATC CCT GAG CTC GAG	45
CGA AGA ATC GAT CAC CGA ATA CAA AAA AGT TGA AAA AGT TAA ACA	90
CGA GGA CCA GGA TCC	105

## (2) INFORMATION FOR SEQ ID NO: 3

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 71 base pairs
- (B) TYPE: Nucleic acid
- (C) STRANDEDNESS: Single
- (D) TOPOLOGY: Unknown
- (ii) MOLECULE TYPE: Synthetic oligonucleotide
  - (A) DESCRIPTION:
- (iii) HYPOTHETICAL:
- (iv) ANTI-SENSE:
- (v) FRAGMENT TYPE:
- (vi) ORIGINAL SOURCE: Synthetic Oligonucleotide Sequence
  - (A) ORGANISM:
  - (B) STRAIN:
  - (C) INDIVIDUAL ISOLATE:
  - (D) DEVELOPMENTAL STAGE:
  - (E) HAPLOTYPE:
  - (F) TISSUE TYPE:
  - (G) CELL TYPE:
  - (H) CELL LINE:
  - (I) ORGANELLE:
- (vii) IMMEDIATE SOURCE:
  - (A) LIBRARY:
  - (B) CLONE:
- (viii) POSITION IN GENOME:
  - (A) CHROMOSOME/SEGMENT:



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(B) MAP POSITION:

(C) UNITS:

(ix) FEATURE:

(A) NAME/KEY:

(B) LOCATION:

(C) IDENTIFICATION METHOD: DNA sequencing and restriction analysis

(D) OTHER INFORMATION: The synthetic oligonucleotide encodes the carboxy-terminal end and translation stop.

(x) PUBUBLICATION INFORMATION:

(A) AUTHORS: L. Hansson, et al.

(B) TITLE: Expression of Human Milk  $\beta$ -casein in Escherichia coli: Comparison of Recombinant Protein with Native Isoforms.

(C) JOURNAL: Protein Expression and Purification

(D) VOLUME: 4

(E) ISSUE:

(F) PAGES: 373 - 381

(G) DATE: 1993

(H) DOCUMENT NUMBER:

(I) FILING DATE:

(J) PUBLICATION DATE:

(K) RELEVANT RESIDUES:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3

AGA TCT ACC CTG TGA CTC AGC CAC TTG CCC CAG TTC ATA ACC CCA  
TTA GTG TCT AAT AAG GAT CCG AAT TC

45

71

(2) INFORMATION FOR SEQ ID NO: 4

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 68 base pairs
  - (B) TYPE: Nucleic acid
  - (C) STRANDEDNESS: Single
  - (D) TOPOLOGY: Unknown
- (ii) MOLECULE TYPE: Synthetic oligonucleotide
  - (A) DESCRIPTION:
- (iii) HYPOTHETICAL:
- (iv) ANTI-SENSE:
- (v) FRAGMENT TYPE:
- (vi) ORIGINAL SOURCE: Synthetic Oligonucleotide Sequence
  - (A) ORGANISM:
  - (B) STRAIN:
  - (C) INDIVIDUAL ISOLATE:
  - (D) DEVELOPMENTAL STAGE:
  - (E) HAPLOTYPE:
  - (F) TISSUE TYPE:
  - (G) CELL TYPE:
  - (H) CELL LINE:
  - (I) ORGANELLE:
- (vii) IMMEDIATE SOURCE:
  - (A) LIBRARY:
  - (B) CLONE:
- (viii) POSITION IN GENOME:

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- (A) CHROMOSOME/SEGMENT:
- (B) MAP POSITION:
- (C) UNITS:
- (ix) FEATURE:
  - (A) NAME/KEY:
  - (B) LOCATION:
  - (C) IDENTIFICATION METHOD: DNA sequencing and restriction analysis
  - (D) OTHER INFORMATION: Modified enterotoxin STII signal sequence.
- (x) PUBUBLICATION INFORMATION:
  - (A) AUTHORS: L. Hansson, et al.
  - (B) TITLE: Expression of Human Milk  $\beta$ -casein in Escherichia coli: Comparison of Recombinant Protein with Native Isoforms.
  - (C) JOURNAL: Protein Expression and Purification
  - (D) VOLUME: 4
  - (E) ISSUE:
  - (F) PAGES: 373 - 381
  - (G) DATE: 1993
  - (H) DOCUMENT NUMBER:
  - (I) FILING DATE:
  - (J) PUBLICATION DATE:
  - (K) RELEVANT RESIDUES:
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4

CAT GAA AAA GAA TAT CGC ATT TCT TCT TGC ATC GAT GTT CGT TTT  
TTC TAT TGC TAC AAA TGC ATA TG

45  
68

- (2) INFORMATION FOR SEQ ID NO: 5

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 37 base pairs
  - (B) TYPE: Nucleic acid
  - (C) STRANDEDNESS: Single
  - (D) TOPOLOGY: Unknown
- (ii) MOLECULE TYPE: Synthetic oligonucleotide
  - (A) DESCRIPTION:
- (iii) HYPOTHETICAL:
- (iv) ANTI-SENSE:
- (v) FRAGMENT TYPE:
- (vi) ORIGINAL SOURCE: Synthetic Oligonucleotide Sequence
  - (A) ORGANISM:
  - (B) STRAIN:
  - (C) INDIVIDUAL ISOLATE:
  - (D) DEVELOPMENTAL STAGE:
  - (E) HAPLOTYPE:
  - (F) TISSUE TYPE:
  - (G) CELL TYPE:
  - (H) CELL LINE:
  - (I) ORGANELLE:
- (vii) IMMEDIATE SOURCE:
  - (A) LIBRARY:
  - (B) CLONE:
- (viii) POSITION IN GENOME:

27

- (A) CHROMOSOME/SEGMENT:
- (B) MAP POSITION:
- (C) UNITS:
- (ix) FEATURE:
  - (A) NAME/KEY:
  - (B) LOCATION:
  - (C) IDENTIFICATION METHOD: DNA sequencing and restriction analysis
  - (D) OTHER INFORMATION:
- (x) PUBUBLICATION INFORMATION:
  - (A) AUTHORS: L. Hansson, et al.
  - (B) TITLE: Expression of Human Milk  $\beta$ -casein in Escherichia coli: Comparison of Recombinant Protein with Native Isoforms.
  - (C) JOURNAL: Protein Expression and Purification
  - (D) VOLUME: 4
  - (E) ISSUE:
  - (F) PAGES: 373 - 381
  - (G) DATE: 1993
  - (H) DOCUMENT NUMBER:
  - (I) FILING DATE:
  - (J) PUBLICATION DATE:
  - (K) RELEVANT RESIDUES:
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5

CAT ATG CAC GTG AAA CCA TCG AAT CCC TGA GCT CGA G

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## CLAIMS:

1. A liquid enteral nutritional product comprising at least one protein not contained in human milk in combination with at least one material selected from the group consisting of  $\beta$ -casein isolated from human milk, a recombinant form of the  $\beta$ -casein contained in human milk and hydrolysates of both in a therapeutically effective amount which inhibits the infection of mammalian cells by RSV.

2. A liquid enteral nutritional product according to claim 1 wherein the product is an infant formula.

3. A liquid enteral nutritional infant formula comprising at least one protein not contained in human milk in combination with at least one material selected from the group consisting of  $\beta$ -casein isolated from human milk, a recombinant form of the  $\beta$ -casein contained in human milk and hydrolysates of both in a therapeutically effective amount which inhibits the infection of mammalian cells by RSV, said infant formula containing no other proteins which are found in human milk.

4. A nasally administrable formulation comprising at least one material selected from the group consisting of  $\beta$ -casein isolated from human milk, a recombinant form of the  $\beta$ -casein contained in human milk and hydrolysates of both in a therapeutically effective amount which inhibits the infection of mammalian cells by RSV.

5. A throat spray formulation comprising at least one material selected from the group consisting of  $\beta$ -casein isolated from human milk, a recombinant form of the  $\beta$ -casein contained in human milk and hydrolysates of both in a therapeutically effective amount which inhibits the infection of mammalian cells by RSV.

6. A method of inhibiting the infection of mammalian cells by RSV by enterally ingesting a liquid nutritional product comprising at least one protein not contained in human milk in combination with a therapeutically effective amount of at least one material selected from the group consisting

of  $\beta$ -casein isolated from human milk, a recombinant form of the  $\beta$ -casein contained in human milk and hydrolysates of both.

7. A method of inhibiting the infection of mammalian cells by *RSV* in a human infant by enterally feeding to said human infant an infant formula comprising at least one protein not contained in human milk in combination with a therapeutically effective amount of at least one material selected from the group consisting of  $\beta$ -casein isolated from human milk, a recombinant form of the  $\beta$ -casein contained in human milk and hydrolysates of both.

8. A method of treating and preventing respiratory tract infection in a human by inhibiting the infection of mammalian cells by *RSV* by feeding to said human an enteral nutritional product comprising at least one protein not contained in human milk in combination with a therapeutically effective amount of at least one material selected from the group consisting of  $\beta$ -casein isolated from human milk, a recombinant form of the  $\beta$ -casein contained in human milk and hydrolysates of both.

9. A method of treating and preventing respiratory tract infection in a human infant by inhibiting the infection of mammalian cells by *RSV* by feeding to said human infant an enteral formula comprising at least one protein not contained in human milk in combination with a therapeutically effective amount of at least one material selected from the group consisting of  $\beta$ -casein isolated from human milk, a recombinant form of the  $\beta$ -casein contained in human milk and hydrolysates of both.

10. A method of inhibiting the infection of mammalian cells by *RSV* by administering via a nasal passageway a formulation containing a therapeutically effective amount of at least one material selected from the group consisting of  $\beta$ -casein isolated from human milk, a recombinant form of the  $\beta$ -casein contained in human milk and hydrolysates of both.

11. A method of inhibiting the infection of mammalian cells by *RSV* by administering a throat spray formulation containing a therapeutically effective amount of at least one material selected from the group consisting

of  $\beta$ -casein isolated from human milk, a recombinant form of the  $\beta$ -casein contained in human milk and hydrolysates of both.



# INTERNATIONAL SEARCH REPORT

Inter. Application No  
PCT/US 95/03628

A. CLASSIFICATION OF SUBJECT MATTER  
IPC 6 A61K38/17 A23L1/305 C07K14/47

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C07K A23L

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO,A,91 06308 (B.ANDERSSON ET AL.) 16 May 1991 cited in the application see the whole document ---	1-11
X	EP,A,0 291 265 (SNOW BRAND MILK PRODUCTS CO. LTD.) 17 November 1988 see claims 1-7 ---	1-11
A	MICROB.PATHOG., vol.8, no.5, May 1990 pages 315 - 323 G.ANIASSON ET AL. 'Anti-adhesive activity of human casein against Streptococcus pneumoniae and Haemophilus influenzae' cited in the application see the whole document --- -/--	1-11

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

### \* Special categories of cited documents :

- \* "A" document defining the general state of the art which is not considered to be of particular relevance
- \* "E" earlier document but published on or after the international filing date
- \* "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- \* "O" document referring to an oral disclosure, use, exhibition or other means
- \* "P" document published prior to the international filing date but later than the priority date claimed

\* "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

\* "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

\* "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

\* "&" document member of the same patent family

Date of the actual completion of the international search

24 July 1995

Date of mailing of the international search report

08.08.95

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2  
NL - 2280 HV Rijswijk  
Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl,  
Fax (+ 31-70) 340-3016

Authorized officer

Theuns, H

# INTERNATIONAL SEARCH REPORT

International Application No  
PCT/US 95/03628

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	ADV.EXP.MED.BIOL., vol.310, 1991 pages 167 - 171 C.SVANBORG ET AL. 'ANTI-ADHESIVE MOLECULES IN HUMAN MILK' see the whole document ---	1-11
X	WO,A,91 08675 (C.W.SLATTERY ET AL.) 27 June 1991 see page 1 - page 3 ---	1-5
X	WO,A,94 06306 (NEW ZEALAND DAIRY RESEARCH INSTITUTE) 31 March 1994 see claims 38,39 ---	1-5
X	WO,A,93 04171 (SYMBICOM AKTIEBOLAG) 4 March 1993 see claims 40-44 ---	1-5
X	WO,A,93 04172 (SYMBICOM AKTIEBOLAG) 4 March 1993 see page 37 - page 42 -----	1-5

# INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 95/03628

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9106308	16-05-91	SE-B- 465109 AU-B- 633647 AU-A- 6647090 EP-A- 0454813 JP-T- 4503077 SE-A- 8903625	29-07-91 04-02-93 31-05-91 06-11-91 04-06-92 01-05-91
EP-A-0291265	17-11-88	JP-A- 63284133 US-A- 5147853 US-A- 5344820	21-11-88 15-09-92 06-09-94
WO-A-9108675	27-06-91	AU-A- 7177391	18-07-91
WO-A-9406306	31-03-94	AU-B- 4987393 EP-A- 0661927	12-04-94 12-07-95
WO-A-9304171	04-03-93	WO-A- 9304172 EP-A- 0599978	04-03-93 08-06-94
WO-A-9304172	04-03-93	WO-A- 9304171 EP-A- 0599978	04-03-93 08-06-94

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 95/03628

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:  
Remark: Although claims 6-11 are directed to a method of treatment of the human/animal body the search has been based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.